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Limits of the morphometric method for field identification of water frogs

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Taxonomic identification of the water frogs has evolved since hybridogenesis has been revealed within the *Rana esculenta* complex. Although the study of protein polymorphism has proved robust in taxonomic information, morphometric measurements are currently used despite of some limitations of the method. By comparing results obtained with these two techniques, this study shows that morphometry is not always decisive for field identification. In the three populations studied, in the mid-Rhône floodplain, the morphs of *Rana ridibunda* and the hybrid *Rana* kl. *esculenta* greatly overlap in morphometric characters.

INTRODUCTION

The Palearctic water frog group is composed of several species (for a review see DUBOIS & OHLER, 1995) and is characterized by three hybridogenetic complexes (synkleptons sensu POLLS-PELAZ, 1989). The *Rana esculenta* complex, which is widespread in central Europe, is the more studied of these complexes. The three taxa of this synklepton (*Rana ridibunda*, *Rana lessonae* and the hybridogenetic hybrid *Rana* kl *esculenta*) have been distinguished by several morphological characters for a long time (e.g. CAMERANO, 1884), but the systematics of water frogs remained confused until the existence of a hybrid complex was demonstrated (BERGER, 1968) In this context, the morphometric indices proposed by BERGER (1966) to discriminate three morphs among the hybridogenetic complex strongly contributed to the systematics of the group, and this method is still commonly used (for a recent review, see OGIELSKA, 1995).

Nevertheless, several morphometric investigations showed an overlap among the characteristic morphs of several taxa (e.g. GÜNTHER et al., 1991; POLLS-PELAZ, 1991; RYBACKI, 1995). Besides using the morphological indices proposed by BERGER (1966), some authors applied sophisticated analysis (discriminant analysis, multivariate analysis) to maximize the morphological differences between taxa (e.g. UZZELL & HOTZ, 1979, PLÖTNER et al., 1994). Despite the increasing complexity of taxonomic identification on the basis of morphometric variables, this morphometric method still remains. On the other hand, the analysis of protein polymorphism proves robust in taxonomic identification.

Although the use of quantitative morphological traits fails in identification of water frogs in eastern France (JOLY et al., 1995, TUNNER, personal communication), some studies only used the morphometric method in frog taxonomy. Because of large number of individuals to be identified, field studies need simple methods. In this context, the aim of this paper was to compare the simplest morphometric measurements currently used (e.g. Dp/Cint) with the analysis of allozymic markers.

MATERIAL AND METHODS

SITES AND SAMPLE SIZES

Three populations (Morte-de-la-Barre, Jons, Pierre-Bénite) were investigated in sites located near the active channel of the Rhône river. The former two ponds are gravel-pits while the last one is a regularly overflowed side arm of the Rhône. The sample size is the following: Pierre-Bénite, n = 28 (15 males and 13 females); Jons, n = 31 (19 males and 12 females); Morte-de-la-Barre, n = 33 (25 males and 8 females). Voucher numbers are: Jo26-33, Jo35, Jo37-38, Jo40-47, Jo55, Jo92-102, PB50-54, PB103-125, MB56-63, MB65-71, MB74-91, all deep-frozen carcasses, kept in our laboratory (Université Lyon 1, France).

PROTEIN ELECTROPHORESIS

Electrophoresis was performed on skeletal muscles. Tissue samples were crushed in a 1.2 g Tris + 0.37 g EDTA + 1 l H_2O + 50 ml NADP 1 % solution. Migration was performed in a Tris citrate gel at pH 6 during 3 to 5 hours under 180 Volts. Tris citrate gel composition was: 48 g starch (12 %), 1.4 ml buffer 1 × (composition of the 10 × buffer: Tris 270 g, citric acid 181 g, H_2O 1000 ml), 398.6 ml H_2O Staining solutions were prepared using modifications of standard procedures (PASTEUR et al., 1987, HOTZ, unpublished).

Four loci were analyzed for somatic tissues: lactate deshydrogenase (LDH-1, Enzyme Commission 1.1.1.27), mannose-phosphate-isomerase (MPI, E C 5.3.1 8), phosphoglucomutase (PGM-2, E C. 2.7.5 1) and creatine kinase (CK, E.C 2.7.3.2). These enzymes were chosen because they are known to be efficient for taxonomic identification of several species and hybrids of water frogs (for review, see HOTZ, 1983 and BEERLI, 1994).

Reference specimens from the collection of the Zürich University (H HOTZ) were used as control samples (2 specimens for each of the following taxa): *Rana perezi* (Elvo Delta, Spain), *Rana* kl. *grafi* (Pouzolles, France), *Rana ridibunda* (Mosina, Poland), *Rana* kl. *esculenta* (Hellberg, Switzerland) and *Rana lessonae* (Poznan, Poland and Hellberg, Switzerland). Respective voucher numbers are. 17027, 17030, 17570, 17572, 18095, 18096, 18011, 18109, 18094, 18102, all deep-frozen tissues (no carcasses), kept in the Zürich University (Switzerland).

MORPHOMETRY

The method of SAGNES (1995) was used in collecting morphometric data Demedulated animals were disposed on a box, near a scale A photograph taken using a video camera was numerized by the computer Using the "Image © software", we scaled the photographs and the variables were measured (fig. 1) Because this software allows to zoom a part of the photograph for measuring variables of small size (the metatarsal tubercle in our study), the

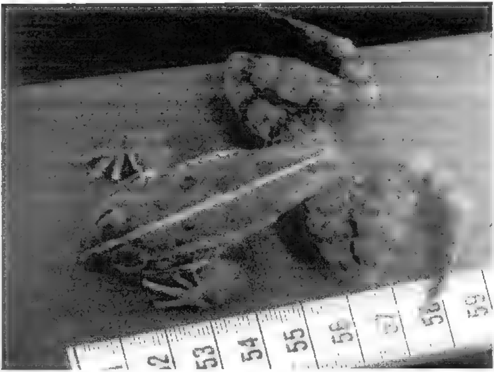

Fig. 1. A specimen of water frog numerized and measured by computer software.

errors in measuring parameters were minimized (Sagnes, 1995). Five variables were measured on computerized frog photographs: Lc (body length), Ti (Tibia length), Dp (First toe length) Cint (Metatarsal tubercle length) and Cint-a (Metatarsal tubercle height). These measurements were used to calculate morphometric indices (Dp/Cint, Ti/Cint, Ti/Cint-a) that are known to discriminate the three forms of the *esculenta* synklepton (Berger, 1966). Male and female analyses were done separately. Measurements were made before freezing the animals.

Results

Electrophoretic identification

The analysis of specific markers in the loci studied established the presence of *Rana ridibunda* and *R.* kl. *esculenta*, and the absence of *R. lessonae*, *R. perezi* and *R.* kl. *grafi* in the sites studied (tab. 1).

Whereas the Jons population was exclusively composed of *R. ridibunda*, the others were mixed populations of *R. ridibunda* and *R.* kl. *esculenta* with 12 % and 19 % of hybrids in Morte-de-la-Barre and Pierre-Bénite, respectively.

Table 1 Specific allozymes or specific genotypes which allow taxonomic identification of water frogs.

Allozymes or genotypes				Species	Number of frogs per site		
LDH-B	MPI	PGM-2	CK-A		Pierre Bénite	Jons	Morte Barre
Allozyme a or c	Allozyme a or c	Allozyme b or d	(1)	*Rana ridibunda*	25	31	29
Genotype ae or ce	Genotype ah	Genotype cd	(1)	*Rana* kl. *esculenta*	6	0	4
Allozyme i or d	Allozyme l or m	(2)	Allozyme d	*Rana perezi*	0	0	0

(1) No specific marker between *R. lessonae* and *R. ridibunda*. The identification of *R.* kl. *esculenta* is not possible with only this locus.
(2) No specific marker between *R. perezi* and *R. ridibunda*.

MORPHOMETRIC IDENTIFICATION

The graph Dp/Cint versus Ti/Cint usually discriminates the different forms of the *esculenta* synklepton (BERGER, 1966). However, in the populations studied and with the morphometric method used (based on computerized photographs), these morphological indices did not clearly separate the different morphotypes neither for males nor for females (fig. 2). Thus, for males, the use of genetic taxonomic markers revealed that the morphological indices of *R.* kl *esculenta* widely overlapped those of *R. ridibunda* in the populations studied (fig. 2), and most of the hybrids could not be distinguished from *R. ridibunda* using these indices. Whereas an overlapping was also evidenced for females, the small sample size does not allow a decisive conclusion

DISCUSSION

In central and eastern Europe, each taxon of the *R. esculenta* synklepton can be identified by several morphological indices (BERGER, 1966, BLANKENHORN et al., 1971,

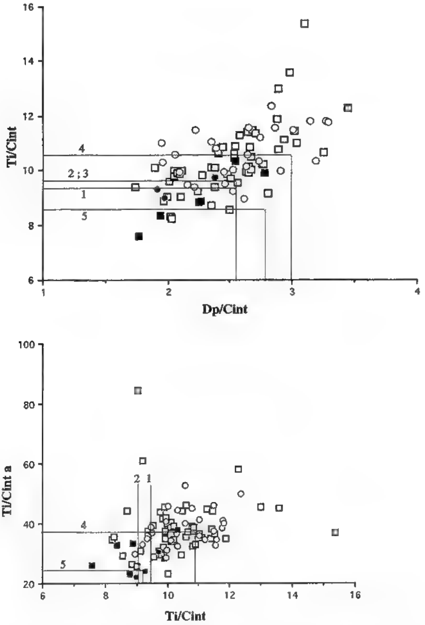

Fig. 2. – The usual graphs Ti/Cint versus Dp/Cint and Ti/Cint versus Ti/Cint-a reveal a great overlapping between the morphotypes of *R.* kl. *esculenta* (black) and *R. ridibunda* (white). Squares symbolize males and circles females. Several thresholds are represented. These limits discriminate *R.* kl. *esculenta* from *R. ridibunda* in the following respective references: (1) Berger, 1966; (2) Colganiceanu & Tesio, 1993; (3) Polls-Pelaz, 1991; (4) Régnier & Neveu, 1986; (5) Wijnands & Van Gelder, 1976. None of these references make it possible to identify the frogs of the present sample.

WIJNANDS & VAN GELDER, 1976). However, in several studies, morphological identification did not correspond with genetic identification (e.g. GÜNTHER et al., 1991; POLLS-PELAZ, 1991; RYBACKI, 1995) In our study, morphological features of *esculenta* males greatly overlapped with those of *R. ridibunda* and no clear morphotype (as currently described) was detected. Thus the morphometric indices are not always valid for taxonomic identification in the field. Morphometric identification is far from being secure, at least in the studied region and using our method (photographs of non-fixed animals). Other studies evidenced similar problems of taxonomic identification (JOLY et al , 1995; KOTLIC & SULOVA, 1995; LADA et al., 1995; RYBACKI, 1995, MORAND et al., in preparation). Thus, the limitations of identification using these indices are striking when we report the values of Ti/Cint given by several authors as discriminating values for the three morphs of the *R. esculenta* synklepton. Thresholds vary between studies (see tab. 2 for a review and fig 2). Though it may be argued that there are artefactual differences linked to differences in methods (fixed specimens or living frogs, differences in measurement methods, investigations with or without taking care of morphometric differences between males and females), such a variation in morphological traits suggests several other hypotheses or questions:

(1) Are morphological traits more representative of adaptation than of phylogenetic relationships? Some ecological variables in relation to a gradient of flood disturbance lead to this hypothesis (MORAND et al., in preparation). The sites we studied were within a floodplain where ecological successions are rapid and different habitats patchily distributed. In tadpoles, variation in size is greater in unpredictable environments than in predictable ones (WILBUR & COLLINS, 1973). Morphology is probably determined on the one hand by phylogenetic constraints and on the other hand by environmental conditions. The absence of distinct morphotypes can be explained by the expression of phenotypic diversity in the context of unpredictable and heterogeneous environments. So, we hypothesize that morphological discrimination found in several studies in stable environments is perhaps more an effect of different, separate and stable habitats than the result of phylogenetic lineage. However, there is no evidence in the literature to support this statement because of a lack of ecological description of sites (PAGANO et al., in preparation). Morphometric method was more used as a taxonomic tool than for ecological investigations. In a same taxon, the morphological variation between populations of different biogeographic regions (tab. 2) can be the result of genetic structurations. Several studies have shown that *R. ridibunda* is highly variable (HOTZ et al., 1985; BEERLI, 1994; PAGANO et al., 1997). Besides, the genetic distance between *R.* kl *esculenta* of France and central Europe is unknown. The hypothesis of genetic structuration within a taxon remains to be tested

(2) According to GROSSENBACHER (1988), the presence of *R. ridibunda* in the upper-Rhône river is recent and due to introductions. In this respect, we can hypothesize that, for a long time, *R.* kl. *esculenta* lived alone in habitats favorable for *R. ridibunda*. So its morphology may reflect its adaptation to these habitats. The absence of distinct morphotypes for *R. ridibunda* and *R.* kl. *esculenta* could be explained by convergence.

(3) Does temperature influence morphological variation? REPA (1977) showed that tibia length was related to the mean water temperature of the ponds. The epigenetic origin of morphological variation has to be studied Such an idea has been suggested to explain the high values of indices in water frogs from western France (RÉGNIER & NEVEU, 1986).

Table 2. – Differences in the discriminating values of the index Ti/Cint for the identification of water frogs in some countries of Europe.

References	*Rana lessonae*	*Rana* kl. *esculenta*	*Rana ridibunda*	Country
RÉGNIER & NEVEU, 1986	< 9.5	9 - 10.4	—	France (Bretagne, North-East)
POLLS-PELAZ, 1991	< 8	8 - 9.5	—	France (Paris region)
GÜNTHER, 1975	< 7	6.5 - 8.6	—	Germany
WIJNANDS & VAN GELDER, 1976	< 6	6 - 8.5	> 8.5	Netherlands
BERGER, 1966	< 7	7 - 9	> 9.5	Poland
COGALNICEANU & TESIO, 1993	< 7	7 - 9.5	> 9.5	Romania

In several studies, investigations were performed on the basis of the sole morphometric identification, but we assert that such an identification is far from being secure. For the moment, only genetic identification provides decisive criteria for taxonomic identification.

Because several studies (experimentation, field studies, etc) need identification of living animals, we may recommend the use of electrophoresis. It is possible to perform such an analysis on a small piece of tissue (a cut toe or blood; HOTZ, personal communication; PAGANO, unpublished data), so that data collection is easy in the field. However, other morphological criteria allowing identification may be found, such as the shape of the vomerine teeth (CROCHET et al., 1995), though the pertinence of such methods has to be checked by extensive comparison with electrophoretic data.

RÉSUMÉ

Pour des raisons historiques, la morphométrie est couramment utilisée pour la détermination taxinomique des grenouilles vertes du complexe *Rana esculenta*. L'utilisation de l'éléctrophorèse de protéines est souvent utilisée à des fins identiques. Dans cette étude, la détermination des spécimens a été effectuée à la fois par l'analyse du polymorphisme enzymatique et par la morphométrie en analyse d'images, contribuant à montrer que cette dernière technique n'est pas totalement fiable pour des determinations sur le terrain.

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